IL-4 receptor engagement in human neutrophils impairs their migration and extracellular trap formation

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Abstract: Background Type 2 immunity serves to resist parasitic helminths, venoms, and toxins, but the role and regulation of neutrophils during type 2 immune responses are controversial. Helminth models suggested a contribution of neutrophils to type 2 immunity, whereas neutrophils are associated with increased disease severity during type 2 inflammatory disorders, such as asthma. Objective We sought to evaluate the effect of the prototypic type 2 cytokines IL-4 and IL-13 on human neutrophils. Methods Human neutrophils from peripheral blood were assessed without or with IL-4 or IL-13 for (1) expression of IL-4 receptor subunits, (2) neutrophil extracellular trap (NET) formation, (3) migration toward CXCL8 in vitro and in humanized mice, and (4) CXCR1, CXCR2, and CXCR4 expression, as well as (5) in nonallergic versus allergic subjects. Results Human neutrophils expressed both types of IL-4 receptors, and their stimulation through IL-4 or IL-13 diminished their ability to form NETs and migrate toward CXCL8 in vitro. Likewise, in vivo chemotaxis in NOD-scid-Il2rg−/− mice was reduced in IL-4–stimulated human neutrophils compared with control values. These effects were accompanied by downregulation of the CXCL8-binding chemokine receptors CXCR1 and CXCR2 on human neutrophils on IL-4 or IL-13 stimulation in vitro. Ex vivo analysis of neutrophils from allergic patients or exposure of neutrophils from nonallergic subjects to allergic donor serum in vitro impaired their NET formation and migration toward CXCL8, thereby mirroring IL-4/IL-13–stimulated neutrophils. Conclusion IL-4 receptor signaling in human neutrophils affects several neutrophil effector functions, which bears important implications for immunity in type 2 inflammatory disorders.

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Interleukin-4 receptor engagement in human neutrophils impairs their migration and extracellular trap formation

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Abstract

Background: Type 2 immunity serves to resist parasitic helminths, venoms and toxins, but the role and regulation of neutrophils during type 2 immune responses is controversial. Helminth models suggested a contribution of neutrophils to type 2 immunity, whereas neutrophils are associated with increased disease severity during type 2 inflammatory disorders such as asthma.

Objective: To evaluate the impact of the prototypic type 2 cytokines interleukin-4 (IL-4) and IL-13 on human neutrophils.

Methods: Human neutrophils from peripheral blood were assessed without or with IL-4 or IL-13 for (i) expression of IL-4 receptor (IL-4R) subunits; (ii) neutrophil extracellular trap (NET) formation; (iii) migration toward CXC chemokine ligand 8 (CXCL8) *in vitro* and in humanized mice; (iv) CXC chemokine receptor 1 (CXCR1), CXCR2, and CXCR4 expression; and (v) in non-allergic versus allergic subjects.

Results: Human neutrophils expressed both types of IL-4Rs and their stimulation via IL-4 or IL-13 diminished their ability to form NETs and migrate toward CXCL8 *in vitro*. Likewise, *in vivo* chemotaxis in NOD-*scid*-*Il2rg*$^{-/-}$ mice was reduced in IL-4-stimulated human neutrophils compared to controls. These effects were accompanied by downregulation of CXCL8-binding chemokine receptors CXCR1 and CXCR2 on human neutrophils upon IL-4 or IL-13 stimulation *in vitro*. *Ex vivo* analysis of neutrophils from allergic patients or exposure of neutrophils from non-allergic individuals to allergic donors' serum *in vitro* impaired their NET formation and migration toward CXCL8, thereby mirroring IL-4/IL-13-stimulated neutrophils.

Conclusion: Signaling in human neutrophils affects several neutrophil effector functions, which bears important implications for immunity in type 2 inflammatory disorders.

Key messages:

- IL-4R stimulation on human neutrophils by IL-4 or IL-13 decreased NET formation.
- Stimulation of IL-4R on human neutrophils downregulated their CXCR1 and CXCR2 and impaired their chemotaxis to CXCL8 *in vitro* as well as in NOD-*scid*-*Il2rg*$^{-/-}$ mice.
- *In vivo* or *in vitro* exposure of neutrophils to allergic patients' serum decreased CXCR1 and CXCR2 as well as impaired NET formation and migration, thereby mirroring IL-4/IL-13-stimulated neutrophils.

Capsule summary: IL-4 receptor engagement in human neutrophils impairs their formation of extracellular traps and their *in vitro* and *in vivo* chemotaxis.

Key words: allergy; neutrophil; innate immunity; IL-4; IL-13; IL-4 receptor; inflammation.

Abbreviations: IL-4R, IL-4 receptor; NET, neutrophils extracellular trap.

Introduction

Neutrophil granulocytes (neutrophils) are the first non-resident immune cells to react to pathogen- or danger-associated stimuli and they rapidly migrate to the site of inflammation. Migration of neutrophils is governed by the expression of the chemokine receptors CXC chemokine receptor 1 (CXCR1) and CXCR2 versus CXCR4. Thus, downregulation of CXCR4 and upregulation of CXCR2 on neutrophils maturing in the bone marrow allows these cells to leave the organ and join the migratory pool of blood neutrophils.[1-3] On the contrary, senescent or non-migratory neutrophils upregulate CXCR4. In the target tissue, neutrophils employ different effector functions, including phagocytosis and the secretion of cytotoxic granules and cytokines. Moreover, activated neutrophils can initiate a cellular program leading to the release of neutrophil extracellular traps (NET) to immobilize and kill large microbes.[4,5] NETs are web-like structures composed of decondensed mitochondrial or nuclear DNA that are associated with modified histone proteins and different antimicrobial peptides, such as neutrophil elastase and myeloperoxidase (MPO). Neutrophils release NETs in response to a range of stimuli, including phorbol 12-myristate 13-acetate (PMA), CXCL8 (also termed interleukin-8 [IL-8]), crystals, lipopolysaccharide (LPS), and microorganisms. NETs not only contribute to pathogen defense, but they are also associated with non-infectious disorders, including vasculitis and systemic lupus erythematosus.

Neutrophils are predominant in type 1 (or T helper [Th] 1 cell) and type 3 (Th17 cell) immune responses. However, the regulation and role of neutrophils during type 2 (or Th2 cell) immune responses remains ill-defined.[6-9] Type 2 immune responses evolved to protect the host against large extracellular parasitic helminths, venoms, and toxins by strengthening epithelial barrier defenses and stimulating

88 different immune cell mechanisms.[10,11] Many of these effects are initiated, driven and
89 maintained by the actions of type 2 cytokines, most notably IL-4 and IL-13, which
90 signal via heterodimeric IL-4 receptors (IL-4R). IL-4 binds and signals via two
91 different IL-4Rs, termed type 1 and type 2 IL-4R, respectively (**Fig. 1A**). The type 1
92 IL-4R consists of a heterodimer made of IL-4Rα (also termed CD124) and the
93 common gamma chain (also known as γ_c or CD132). The type 2 IL-4R is composed
94 of CD124 and IL-13Rα1 (also named CD213α1). IL-13Rα2 (also termed CD213α2)
95 serves as a decoy receptor for IL-13. In addition to IL-4, IL-13 also associates with
96 and signals through the type 2 IL-4R. IL-4 and IL-13 mediate signaling via
97 phosphorylation of Signal Transducer and Activator of Transcription 6 (STAT6),[11,12]
98 but typically not of STAT5 and STAT3, whereas STAT5 and STAT3 become
99 activated with γ_c cytokines and granulocyte colony-stimulating factor (G-CSF).[13]

100 In type 2 immunity, during certain helminth infections in mice, the recruitment
101 of neutrophils contributed to early containment of the parasite during its migration
102 through the lungs.[14] Yet, in the same model, neutrophils also caused increased
103 damage to the lungs.[14,15] Considering type 2 inflammation in humans, as typically
104 seen with atopic dermatitis and allergic asthma, some studies have shown that
105 neutrophil responses were attenuated or maybe even suppressed, which has been
106 attributed to reciprocal regulation of type 2 and type 3 inflammatory pathways.[16,17]
107 However, neutrophils are present in certain severe forms of allergic asthma, which
108 may result in a mixed type 2 and type 3 inflammation,[6,18-21] and NETs have recently
109 been associated with asthma exacerbations during rhinovirus infection of asthmatics.[22]
110 Notably, coding polymorphisms in the human *IL4Ra* gene have been linked to
111 susceptibility and severity of atopy and asthma in patients,[23-25] as well as a mixed
112 neutrophilic-eosinophilic type of severe experimental asthma in mice.[26] Moreover,

recently, mice with a deficiency in IL-4, IL-13 or STAT6 were shown to exhibit a neutrophilic form of experimental asthma upon challenge.[27]

These data suggest that the IL-4R–STAT6 signaling pathway regulates neutrophils in type 2 inflammation, possibly via other, more rapid mechanisms than its involvement in type 2 vs. type 3 immune skewing. With the hypothesis that IL-4 could directly affect neutrophils, as recently shown in mice,[28] we have investigated in the present study expression and impact on primary human neutrophils of IL-4R signaling following stimulation with the prototypic type 2 cytokines IL-4 and IL-13.

Materials and Methods

Human subjects

Following written informed consent, volunteers were recruited for donating blood, which was immediately processed to isolate neutrophils. Serum was also obtained from the same donors for IgE detection using ImmunoCAP250 (Phadia, ThermoFisher). All experiments using human samples were carried out in accordance with the Cantonal Ethical Committee of Zurich (BASEC number 2016-01440). Healthy donors (HD) were defined as subjects with serum IgE levels below 100 kU/L and no history of seasonal or perennial allergies. Allergic donors (AD) were subjects with total serum IgE levels above 100 kU/L (mean 279.7 ± 184.9 kU/L) with a known allergy to grass pollen, tree pollen, or house dust mite, verified by a positive skin prick test or allergen-specific IgE test, and with specific clinical symptoms such as seasonal or perennial rhino-conjunctivitis and asthma. AD were investigated during their allergen season. Absolute neutrophil counts and percentages in peripheral blood were determined by an automated system (Abbott Diagnostics, Santa Clara).

Isolation and *in vitro* stimulation of neutrophils

Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer®). Neutrophils were purified by HetaSep™ (Stemcell Technologies) followed by negative magnetic selection using the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies). Cell viability (>97%) was assessed by trypan blue exclusion and purity of neutrophil preparations (96.4 ±2.4%) was determined by flow cytometry based on CD16 (3G8, BioLegend), CD61 (VI-PL2, BioLegend) and CD66b (G10F5, BioLegend). Purified neutrophils were resuspended in RPMI 1640 supplemented with 1% fetal bovine serum (FBS;

ThermoFisher), before stimulation with recombinant human cytokines (used throughout the study), including G-CSF, IL-4, and IL-13 (at 150 ng/mL, unless stated otherwise; PeproTech), for 0, 4, 6, and 24 h, or with serum from either HD or AD, followed by flow cytometry analysis (see below). Viability was measured using the Annexin V Apoptosis Detection kit (BD Biosciences) in combination with propidium iodide (PI).

Flow cytometry

Single-cell suspensions of neutrophils were processed for analysis by flow cytometry and stained using phosphate-buffered saline (PBS) with 1% FBS, 2 mM EDTA, and fluorochrome-conjugated monoclonal antibodies against the following human antigens (from BioLegend unless otherwise stated): CD16 (3G8), CD61 (VI-PL2), CD66b (G10F5), CD114 (LMM741), CD124 (G077F6), CD132 (TUGh4), CD162 (KPL-1), CD213α1 (SS12B), CD213α2 (SHM38), CXCR1 (8F1/CXCR1), CXCR2 (5E8/CXCR2), and CXCR4 (12G5). For MPO staining, neutrophils were fixed by addition of Fixation Buffer I, followed by permeabilization with Perm Wash Buffer III (BD Phosflow, BD Bioscience). Intracellular staining for MPO (MPO421-8B2) was performed according to manufacturer's instructions. Samples were acquired using a BD LSR Fortessa and analyzed using FlowJo software (Tristar).

Assessment of intracellular signaling pathways

Highly-purified neutrophils were stimulated *in vitro* in RPMI medium (RPMI 1640 with 1% of FBS) and G-CSF, IL-4, or IL-13 (50 ng/mL) for 0, 2, 5, 15, 30, and 60 min, followed by fixation with Fix Buffer I and permeabilization with Perm Buffer III. Intracellular staining for phospho-Y705 of STAT3 (pSTAT3; 13A3-1,

BioLegend), phospho-Y694 of STAT5 (pSTAT5; SRBCZX, ThermoFisher), phospho-Y641 of STAT6 (pSTAT6; CHI2S4, ThermoFisher) was performed as previously established.[28,29] Samples were acquired and analyzed by flow cytometry, as stated above.

SDS-PAGE and Western blot analysis

10^7 neutrophils in RPMI 1640 supplemented with 1% FBS were stimulated with G-CSF, IL-4, or IL-13 (50 ng/mL) for 0 and 5 min at 37°C. Subsequently, cells were lyzed with 4x Laemmli's buffer (Bio-Rad). Proteins were separated using 4–20% SDS-PAGE mini ready gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were labeled with rabbit antibody (Cell Signaling Technology) for pSTAT3 (D3A7), pSTAT5, pSTAT6, total STAT3 (79D7), total STAT5 (D206Y), total STAT6 (D3H4), and vinculin. After incubation with the appropriate primary antibody, the blots were developed with horseradish peroxidase-conjugated secondary antibodies by using enhanced chemiluminescence reagents (ThermoScientific) following standard protocols.

Analysis of NETs

10^5 neutrophils were seeded on glass coverslips treated with poly-lysine (Sigma) in 24-well tissue culture plates and incubated for 6 h at 37°C in 5% CO_2 with medium (RPMI 1640 with 1% of FBS) alone or with G-CSF, IL-4, or IL-13 (150 ng/mL). Subsequently, neutrophils were stimulated with 100 nM PMA (Sigma) for 2 h to induce NET formation. Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and mounted using ProLong Gold AntiFade with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies). Cells were imaged using

198 a 40-fold magnification 1.25 NA with an inverted CSLM Leica SP5 confocal
199 microscope (Leica). Every picture consisted of an overlay of 15 stacks of 20 μm.
200 Quantification of NET^+ neutrophils were done both manually and automatically using
201 ImageJ software. For manual counting, 5 randomly selected areas of the same slide
202 were counted by three independent investigators with two of them blinded to the
203 treatment conditions. For automatic analysis, we used ImageJ software with the
204 DANA plug-in to quantify the area, raw integrated density, aspect ratio, roundness,
205 maximum and minimum brightness, and solidity of each region of interest of DAPI-
206 labeled neutrophils, as previously described.[30]
207
208 **Transwell migration assay**
209 Transwell migration assays were performed as previously published.[28,31] In brief,
210 freshly-isolated neutrophils were incubated for 2 h at 37°C in 5% CO_2 with medium
211 (RPMI 1640 containing 1% of FBS) alone or supplemented with 150 ng/mL G-CSF,
212 IL-4, IL-13, or serum from HD or AD. Where indicated, neutrophils were incubated
213 with cytokines, or with serum, or preincubated with rabbit anti-human IL-4Rα
214 monoclonal antibody (anti-CD124; Sino Biological) for 1 h before seeding into the
215 upper chamber of a 3 μm transwell (Corning Costar). Neutrophil migration toward
216 CXCL8 (PeproTech), added to the lower chamber, was determined after 120 min by
217 determining neutrophil counts in the lower chamber using flow cytometry counting
218 beads (123count eBeads, ThermoFisher), as previously published.[32,33]
219
220 **Airpouch mouse model**
221 An airpouch was formed in the back of 2–4-month-old NOD-*scid*-*Il2rg*$^{-/-}$ (NSG;
222 Charles River Laboratories) mice by subcutaneous injection of 3 mL filtered sterile air

on days 0 and 3, as described.[28] On day 5, LPS (100 nM) and CXCL8 (100 ng/mL) in 2 mL of PBS was injected into the airpouch along with intravenous injection of 10^7 highly-purified human neutrophils pre-incubated for 2–6 h with medium (RPMI 1640 medium with 1% FBS) alone or in combination with IL-4 (150 ng/mL), or G-CSF (150 ng/mL). Mice were euthanized after 90 min to collect airpouch infiltrate and spleens. Samples were analyzed by flow cytometry as stated above. Mouse experiments followed the Swiss Federal Veterinary Office guidelines and were approved by the Cantonal Veterinary Office.

Statistics

The numbers of samples and subjects used in each experiment are indicated in the figure legends. Data are presented as mean ± standard deviations (SD). *P*-values were calculated using Student's *t*-test, two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test, one-way ANOVA with Tukey's multiple comparisons test, or Friedman test with Dunn's multiple comparisons test as indicated. Statistical analysis was performed with Graph-Pad Prism. Statistical significance was established at $P < 0.05$.

Results

Human neutrophils express functional type 1 and type 2 IL-4Rs

Previous studies found human neutrophils expressed both CD124 and CD132, thus forming type 1 IL-4Rs, whereas CD213α1 and CD213α2 were absent on these cells.[34,35] We re-evaluated IL-4R expression on freshly-isolated human neutrophils. Notably, our neutrophil purification method, yielding on average 96.4% pure human neutrophils, did not significantly affect expression levels of CD16, CD66b, IL-4R subunits, crucial chemokine receptors, as well as CD49d, in comparison to the assessment of these molecules on neutrophils in whole blood (**Fig. E1, A** to **C**). We were able to detect CD124, CD132, and CD213α1 on purified human neutrophils right after isolation from whole blood of HD, and expression of these IL-4R subunits further significantly increased upon stimulation of neutrophils for 24 h with G-CSF ($P < .0001$, CD124 for G-CSF vs time 0; $P < .0001$, CD132 for G-CSF vs time 0; $P = .0008$, CD213α1 for G-CSF vs time 0; **Fig. 1, B** to **D**). Also, the decoy receptor CD213α2 was detectable on neutrophils and increased upon incubation of neutrophils with G-CSF ($P = .0385$, G-CSF vs time 0; **Fig. E1B** and **E2**). A resting period of 30 to 120 min in medium or autologous serum following isolation did not change IL-4R expression (data not shown).

To investigate whether neutrophils expressed functional IL-4Rs resulting in phosphorylated STAT6 (pSTAT6), we performed time-course experiments in purified human neutrophils upon stimulation with IL-4 or IL-13, and G-CSF as a control. G-CSF resulted in strong and significant activation of STAT3 ($P < .0001$) and STAT5 ($P = .0031$) in human neutrophils 5 min after stimulation, whereas such treatment did not affect pSTAT6 levels (**Fig. 1E**). On the other hand, IL-4 and IL-13 significantly

activated STAT6 in human neutrophils, inducing a sharp peak in pSTAT6 5 min after cytokine stimulation ($P < .0001$, for both IL-4 and IL-13 at 5 min vs time 0), which declined thereafter (**Fig. 1E**). However, pSTAT3 and pSTAT5 expression did not change significantly following IL-4 or IL-13 stimulation (**Fig. 1E**). These findings were further confirmed by Western blot analysis (**Fig. E3**).

Together, these results demonstrate the presence of functional type 1 and type 2 IL-4Rs on human neutrophils.

IL-4R engagement impairs formation of NETs in human neutrophils

To evaluate the effects of IL-4 and IL-13 on neutrophil effector functions we assessed NET formation upon stimulation with PMA (**Fig. 2**). Compared to medium or G-CSF, IL-4- or IL-13-conditioned neutrophils produced significantly less NETs, as assessed by manual counting ($P < .0001$, Medium vs IL-4; $P = .0001$, Medium vs IL-13; $P < .0001$, G-CSF vs IL-4 or IL-13) and by automatic analysis of DNA area ($P < .0001$, Medium vs IL-4 or IL-13; $P < .0001$, G-CSF vs IL-4; $P = .0037$, G-CSF vs IL-13; **Fig. 2, A** and **B**). Moreover, the NETs of IL-4-/IL-13-stimulated neutrophils were of different characteristics featuring enlarged and less dense nuclei next to groups of round and condensed nuclei (**Fig. 2A**). These phenotypic differences were also responsible for the slight but non-significant difference in manual counting vs automatic analysis of NETs in medium-conditioned neutrophils (**Fig. 2B**). These data establish that IL-4R stimulation significantly affects NET formation in human neutrophils.

IL-4 and IL-13 inhibit chemotaxis of human neutrophils to CXCL8 *in vitro*

To gain further insight into the effects of IL-4R stimulation on human neutrophils, we assessed *in vitro* migration of neutrophils using a transwell migration assay. When freshly-isolated human neutrophils were pre-incubated for different times without (Medium) or with IL-4 (**Fig. 3, A** and **B**) or IL-13 (**Fig. 3, C** and **D**), IL-4-/IL-13-stimulated neutrophils showed decreased migration toward CXCL8 (which binds CXCR1 and CXCR2)[3], with significant and consistent reduction in chemotaxis at 60 (P = .0014 [in cell counts] and P = .0043 [in %] for IL-4 vs Medium; P < .0001 and P = .0042, for IL-13 vs Medium) and 120 min (P = .0029 and P = .0004 for IL-4 vs Medium; P < .0001 and P < .0001 for IL-13 vs Medium; **Fig. 3, A** to **D**). Next, we assessed the migration of human neutrophils toward different concentrations of CXCL8 pre-incubating the human neutrophils with IL-4 (**Fig. 3E**) or IL-13 (**Fig. 3F**). While 10 to 100 ng/mL CXCL8 exerted potent chemotactic activity on control neutrophils (medium), pre-incubation with IL-4 or IL-13 significantly decreased neutrophil chemotaxis at all CXCL8 concentrations of 1 ng/mL and higher (P = .0360, IL-4 vs Medium for 1 ng/mL; P = .0263, IL-4 vs Medium for 10 ng/mL; P = .0076, IL-4 vs Medium for 100 ng/mL; P = .0198, IL-13 vs Medium for 1 ng/mL; P = .0377, IL-13 vs Medium for 10 ng/mL; P = .0240, IL-13 vs Medium for 100 ng/mL; P = .0204, IL-13 vs Medium for 1000 ng/mL; **Fig. 3, E** and **F**). Finally, human neutrophils were incubated with different concentrations of either IL-4 or IL-13 followed by chemotaxis toward a set concentration of CXCL8 (P = .0004, 150 ng/mL of IL-4 vs Medium; P = .0002, 150 ng/mL of IL-13 vs Medium). Both IL-4 and IL-13 significantly inhibited neutrophil migration in a dose-dependent manner (**Fig. 3, G** and **H**).

IL-4R signaling hampers migration of human neutrophils in humanized mice

To investigate whether IL-4R signaling inhibited the migration of human neutrophils *in vivo*, we challenged NSG mice harboring human neutrophils. To this end, we induced a sterile inflammation in form of an airpouch on the back of NSG mice by repeated injection of sterile air on days 0 and 3, followed by administration of the neutrophil chemoattractants CXCL8 and LPS into the airpouch and adoptive transfer of pre-conditioned human neutrophils via tail vein injection on day 5 (**Fig. 4A**). Mice were sacrificed 90 min after adoptive transfer of neutrophils to avoid loss of neutrophils, and we analyzed the airpouch infiltrate by flow cytometry by gating on human CD45-positive (and mouse CD45-negative) $CD16^+$ $CD66b^+$ cells (**Fig. 4B**). In the airpouch, the counts of human neutrophils were 4.2 times higher in the control group (Medium) compared to IL-4 (P = .0247; **Fig. 4C**). Conversely, the counts of infiltrating human neutrophils in spleen was comparable in both groups of mice (**Fig. 4C**). Moreover, we compared G-CSF to IL-4, which confirmed that IL-4 pre-conditioning significantly affected migration of human neutrophils into to the airpouch (P = .0214), but not the spleen (**Fig. 4D**).

IL-4R engagement modulates chemokine receptors on human neutrophils

The aforementioned data demonstrate that IL-4R engagement hampers the migration of human neutrophils *in vitro* and *in vivo*. These actions could, at least in part, result from changes in expression of CXCR1, CXCR2 and CXCR4. We assessed expression of these chemokine receptors in human neutrophils upon *in vitro* stimulation with IL-4 or IL-13. As previously mentioned, the isolation method did not affect the expression of these chemokine receptors (**Fig. E1C**). Upon stimulation with either IL-4 or IL-13, human neutrophils significantly downregulated CXCR1 (P = .0077, IL-4

vs Medium; P = .0031, IL-13 vs Medium) and CXCR2 (P = .0238, IL-4 vs Medium; P = .0170, IL-13 vs Medium) compared to incubation in medium (**Fig. 5A**). In contrast, CXCR4 expression did not significantly change in IL-4-/IL-13-conditioned neutrophils (**Fig. 5A**). The changes in CXCR1 and CXCR2 were not due to apoptosis, as shown by annexin V and PI staining, which remained below 5% during the incubation period (**Fig. 5B**). Collectively, IL-4-/IL-13-stimulated neutrophils adopt a chemokine receptor pattern akin to bone marrow-sessile, non-migratory cells.

Neutrophils of allergic individuals resemble IL-4-/IL-13-stimulated cells

The effects of IL-4R signaling on human neutrophils suggest that, in type 2 inflammatory disorders, neutrophil functions could be affected. To investigate this hypothesis, we compared freshly-isolated neutrophils from healthy donors (HD) to patients with known active allergies (see Materials and Methods). Counts and percentages of neutrophils in peripheral blood of allergic donors (AD) were comparable to that of HD (**Fig. 6A**). However, neutrophils from AD exhibited differences in several functional markers, including a significant decrease in CD16 (P = .0071, AD vs HD; **Fig. 6B**). Moreover, neutrophils from AD showed significantly lower expression of CXCR1 (P = .0012, AD vs HD) and CXCR2 (P < .0001, AD vs HD), as well as a tendency toward higher CXCR4 levels (**Fig. 6C**). Thus, neutrophils of AD were reminiscent of IL-4-/IL-13-conditioned cells.

Moreover, the expression levels of MPO in neutrophils from AD were significantly lower compared to HD (P = .0002; **Fig. 6D**). MPO contributes to antimicrobial activity and NET formation.[36] In line with this phenotypic change, neutrophils from AD showed significantly impaired NET formation (P < .0001, AD vs HD) compared to the ones isolated from HD upon stimulation with PMA (**Fig. 6E**).

363 Overall, human neutrophils from AD resemble IL-4-/IL-13-stimulated
364 neutrophils in terms of migratory phenotype and NET formation.
365
366 **Human neutrophils stimulated with serum from allergic individuals show**
367 **similarity with IL-4/IL-13 stimulated cells**
368 To further evaluate whether soluble components of AD serum can affect neutrophils,
369 we incubated freshly-isolated neutrophils from HD with serum from HD or AD.
370 Compared to incubation with HD serum, AD serum severely affected the ability of
371 HD neutrophils to form NETs, as measured by counting ($P < .0001$) and assessment
372 of DNA area ($P = .0056$; **Fig. 7A**). Moreover, the migration of HD neutrophils toward
373 100 ng/mL CXCL8 was significantly reduced upon incubation with AD serum ($P <$
374 .0001), and concomitant use of an anti-CD124 monoclonal antibody was able to
375 partially restore their migration ($P = .0033$, HD neutrophils preincubated with anti-
376 CD124 vs Ctrl followed by AD serum; **Fig. 7B**), indicating that IL-4 and/or IL-13 in
377 AD serum significantly affected the HD neutrophils. These functional changes in HD
378 neutrophils incubated with AD serum were accompanied by a significant decrease of
379 CXCR1 ($P < .0001$) and CXCR2 ($P = .0004$), whereas CXCR4 remained unchanged
380 (**Fig. E4**).
381 Overall, serum from allergic individuals alters the neutrophil chemotaxis and
382 NET formation, thereby mimicking the effects of IL-4 and IL-13.
383

Discussion

Our data demonstrate that the prototypic type 2 cytokines IL-4 and IL-13 adversely affect several functional properties of human neutrophils. As a result, neutrophils appear desensitized toward CXCL8-mediated chemotaxis *in vitro* and *in vivo* as well as to stimulation-induced NET formation. Such IL-4R-mediated conditioning of neutrophils results in a phenotype characterized by low CXCR1 and CXCR2 and decreased MPO expression. These phenotypic and functional characteristics are also observed in freshly-isolated neutrophils from allergic subjects as well as upon incubation of HD neutrophils with AD serum.

Our data suggest that human neutrophils stimulated by their IL-4Rs enter an activation or differentiation state that differs from neutrophils freshly isolated from peripheral blood of healthy individuals. The question arises whether IL-4/IL-13-conditioned neutrophils represent senescent, aged, exhausted or apoptosis-prone cells. We have not observed increased apoptosis of IL-4/IL-13-conditioned neutrophils. Also, unlike senescent or aged neutrophils, showing decreased CD16 and increased CXCR4 and NET formation,[1,37] CXCR4 expression remained unchanged in IL-4/IL-13-conditioned neutrophils, although CD16 was slightly decreased, and they showed a deficiency in NET formation, which is contrary to what was observed for senescent and aged neutrophils.

In terms of NET formation, IL-4/IL-13-conditioned neutrophils could present with a delayed type of NET formation or, alternatively, IL-4R signaling could skew neutrophils toward suicidal vs. vital NET formation.[38-40] Although we did not observe a kinetic delay in NET formation or increased apoptosis in IL-4/IL-13-conditioned neutrophils, we cannot formally exclude these possibilities. We prefer to consider IL-4/IL-13-conditioned neutrophils as "alternatively-activated" neutrophils, similar to

409 their IL-4/IL-13-conditioned counterparts in macrophages.[12] "N2" neutrophils have
410 been described in the context of tumor-infiltrating pro-tumorigenic neutrophils.[41]
411 Whether IL-4/IL-13-conditioned neutrophils resemble N2 neutrophils is an interesting
412 question for future studies.

413 Steady-state and induced levels of IL-4Rs on neutrophils will determine their
414 susceptibility to IL-4 and IL-13. We found that freshly-isolated human neutrophils
415 from whole blood expressed significant levels of type 1 and type 2 IL-4Rs. This
416 finding slightly contrasts with a previous publication describing only the presence of
417 type 1 IL-4Rs on human neutrophils.[35] Yet, another publication reported the absence
418 of type 1 IL-4Rs but the expression of type 2 IL-4Rs on mouse neutrophils.[28] These
419 differences might indicate discrepancies between human and mouse neutrophils, and
420 they might result from different neutrophil preparation methods, such as the
421 preincubation of neutrophils with autologous serum to prevent nonspecific binding via
422 Fc receptors.[35] Importantly, IL-4R expression on human neutrophils was further
423 increased by certain stimuli, most notably G-CSF. This result is consistent with what
424 has been reported in mice.[28] Also, Toll-like receptor (TLR) ligands and common
425 vaccine adjuvants, including LPS, alum and incomplete Freund's adjuvant, have been
426 shown in mice to upregulate CD124 on $CD11b^+$ myeloid cells, which include
427 monocytes and neutrophils.[42] G-CSF and TLR ligands are typically released upon
428 inflammation and infection, indicating that such conditions increase the neutrophils'
429 sensitivity to IL-4 and IL-13.

430 IL-4/IL-13-mediated conditioning of neutrophils, as described here, could play
431 a role in shaping the different asthma phenotypes, including eosinophilic and
432 neutrophilic asthma.[17,43,44] Thus, production of IL-4 and/or IL-13 and the ensuing IL-
433 4R engagement could contribute to the eosinophilic variant of asthma by inhibiting

434 the recruitment of neutrophils into the tissues. This suggestion is in line with mouse
435 data showing that eosinophilic asthma is dependent on IL-4, IL-13, and STAT6,
436 whereas lack of these factors favors neutrophilic asthma in mice.[27] Also, this
437 hypothesis fits with publications reporting that type 2 and type 3 immune signatures
438 are mutually exclusive in asthma patients.[17] Conversely, it is known that rhinovirus
439 infection can result in neutrophil activation and release of NETs along with disease
440 exacerbation in asthmatic subjects.[6,22] Moreover, certain *IL4Ra* genotypes predispose
441 to a mixed eosinophilic-neutrophilic asthma presentation.[23-26] These considerations
442 suggest that IL-4R-mediated conditioning of neutrophils can be overwritten by
443 stronger stimuli, such as an infection, or by interference with normal IL-4R signaling.
444 For eosinophilic asthma, IL-4R-interfering biologic agents should not only
445 dampen type 2 inflammation and improve disease[45,46] but also re-invigorate
446 neutrophils thereby strengthening anti-pathogen immunity. This has so far not been
447 investigated. Contrarily, the use of IL-4R-stimulating molecules could be used in
448 diseases with predominant neutrophilic inflammation, an idea that has been tested in
449 both humans and mice. In psoriasis, a skin and joint disease characterized by
450 prominent neutrophil infiltration, a small proof-of-concept clinical trial showed that
451 administration of recombinant IL-4 improved skin disease, which the authors
452 interpreted as a skewing from Th1 to Th2 cell responses.[47] In mice and rats, provision
453 of recombinant IL-4 improved experimental arthritis,[48,49] which in the collagen-
454 induced DBA/1 mouse model is known to rely on IL-1β and G-CSF-dependent
455 neutrophil activity.[50,51] However, translation and use of recombinant IL-4 in humans
456 is currently hampered by the very short *in vivo* half-life and dose-dependent toxic
457 adverse effects of IL-4, which might be improved by provision of long-acting IL-4
458 formulations.[52]

Collectively, our data demonstrate that IL-4R engagement in human neutrophils antagonizes several effector functions, which might open possibilities to interfere with this pathway in allergic and neutrophilic disorders.

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Author contribution

D.I. designed and performed the experiments, analyzed the data and wrote the manuscript. F.R. performed the experiments. M.R. collected the human samples, analyzed confocal microscopy data and wrote the manuscript. C.E. performed Western blot analysis. A.G.A.K. collected human serum from healthy and allergic donors. J.W. and D.L. gave scientific input. O.B. designed and analyzed experiments, supervised the study, and wrote the manuscript with input from all the authors.

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643
644

Figure legends

Figure 1. Human neutrophils express functional type 1 and type 2 IL-4 receptors. A, Schematic representation of the type 1 and type 2 IL-4R. **B** to **D,** Expression of IL-4R subunits on human neutrophils of healthy donors (HD). Representative histograms of CD124 (**B**), CD132 (**C**), and CD213α1 (**D**) after isolation (T0; black lines) or following stimulation for 24 h with granulocyte colony-stimulating factor (G-CSF; blue lines). Fluorescence minus one (FMO) values are represented by histograms filled with grey color. Bars represent the geometric mean fluorescence intensity (GMFI) of the indicated receptor subunits at T0 (grey bars) or upon 24 h stimulation with G-CSF (blue bars). Data are presented as mean ± standard deviation (SD) of n = 15 donors. Significance of the differences between groups was calculated using Student's t-test. (**E**) STAT pathways activated by G-CSF, IL-4, or IL-13. Phosphorylated STAT3 (pSTAT3; top row), pSTAT5 (middle row), and pSTAT6 (bottom row) in human neutrophils upon stimulation for the indicated times with G-CSF (left column), IL-4 (middle column) or IL-13 (right column). Diagrams represent MFI of pSTATs at indicated time points. Small squares show representative flow cytometry histograms of pSTATs at 0 and 5 min. Data are shown as mean ± SD and are representative of n = 10 independent experiments using unrelated donors. Statistical significance was calculated using Friedman test with Dunn's multiple comparisons test; *ns*, Not significant.

Figure 2. Inhibition of NET formation by IL-4 or IL-13 stimulation *in vitro*. A, Freshly-isolated HD blood neutrophils were directly assessed (Ctrl) or stimulated for 2 h with 100 nM phorbol 12-myristate 13-acetate (PMA) in medium alone (Medium; RPMI 1640 with 1% of FBS) or medium plus granulocyte colony-stimulating factor (G-CSF; 150 ng/mL), IL-4 (150 ng/mL) or IL-13 (150 ng/mL). Neutrophils were stained with DAPI and analyzed by confocal microscopy at 40-fold (40x) and 80-fold (80x) magnification. Experiments were repeated using n = 10 unrelated donors. Scale bar 100 μm. **B,** Bar histograms represent NET^+ neutrophils treated as in **A**, including direct assessment (Ctrl) or upon 2 h PMA stimulation with medium alone (Medium; black filled bar), medium containing G-CSF (150 ng/mL; blue open bar), medium containing IL-4 (150 ng/mL; red filled bar), or medium containing IL-13 (150 ng/mL; blue filled bar). Neutrophils were stained with DAPI and analyzed by confocal microscopy, followed by manual counting of NET^+ neutrophils (left panel) or automatic quantification of the DNA area (μm^2) using the DANA plug-in for ImageJ (right panel). Data are shown as mean ± SD of at least n = 10 independent experiments with unrelated donors. Significance of the differences between groups was calculated using one-way ANOVA; *ns*, Not significant.

Figure 3. Human neutrophil chemotaxis is inhibited by IL-4 and IL-13. A to **D,** Migration of freshly-isolated HD neutrophils toward a set concentration of CXCL8 (100 ng/mL) at different time points (**A** and **C**) or following stimulation for 60 min and 120 min (left and right panels, respectively, **B** and **D**) with medium (black), medium plus IL-4 (150 ng/mL; red; **A** and **B**), or medium plus IL-13 (150 ng/mL; blue; **C** and **D**). **E** and **F**, Chemotaxis of freshly-isolated HD neutrophils toward titrated concentrations of CXCL8 following stimulation for 2 h with set concentrations of IL-4 (150 ng/mL, red; **E**) or IL-13 (150 ng/mL, blue; **F**). **G** and **H**, Migration of freshly-isolated HD neutrophils toward a set concentration of CXCL8 (100 ng/mL) after 2 h of stimulation with titrated concentrations of IL-4 (red filled bars; **G**) or IL-13 (blue filled bars; **H**). Shown are counts (**A**, **C**, **E**, and **F**) or percentages (**B**, **D**, **G**, and **H**) of migrated neutrophils. Data are presented as mean ± SD of three independent experiments with n = 10-12 unrelated donors. Significances between the groups were calculated by one-way ANOVA (**A**, **C**, and **E** to **H**) or Student's t-test (**B** and **D**).

Figure 4. Migration of human neutrophils *in vivo*. A, Experimental setup of the airpouch in NOD-*scid*-*Il2rg*$^{-/-}$ (NSG) mouse model. **B,** Representative flow cytometry analysis of human (h) CD45^{+} mouse (m) CD45^{-} cells (left panel) and hCD16^{+} hCD66b^{+} neutrophils (right panel) in the airpouch. **C,** Bars represent the counts of human neutrophils, on the total of CD45^{+} human cells, preconditioned for 2 h with medium (RPMI 1640 containing 1% of FBS, black bars; n = 8) or with IL-4 (150 ng/mL, red filled bars; n = 10) in the airpouch (left panel) or spleen (right panel). **D,** Counts of human neutrophils preconditioned for 6 h with G-CSF (150 ng/mL, blue empty bars; n = 6) or with IL-4 (red filled bars; n = 6) collected from the airpouch (left panel) or spleen (right panel). Data are presented as mean ± SD of five (**C**) or three (**D**) independent experiments using unrelated HD. Significance of the differences between groups was calculated using Student's *t*-test; *ns*, Not significant.

Figure 5. Modulation of CXC chemokine receptors CXCR1 and CXCR2 on human neutrophils. A, Expression of CXC chemokine receptors CXCR1, CXCR2, and CXCR4 on HD neutrophils following stimulation for 6 h with medium (RPMI 1640 containing 1% of FBS, black filled bars), IL-4 (red filled bars), or IL-13 (blue filled bars). Shown are the GMFI of indicated CXC chemokine receptors. Data are shown as mean ± SD of $n = 10$ donors. Significance of the differences between groups was calculated using one-way ANOVA. **B,** Representative flow cytometry analysis of annexin V and propidium iodide (PI) staining on HD neutrophils treated as in **A**. Numbers in plot indicate the percentage. Experiments were repeated $n = 10$ times with independent, unrelated donors; *ns*, Not significant.

Figure 6. Human neutrophils from healthy versus allergic individuals. A to **D,** Freshly-isolated neutrophils from HD (black open bars) and allergic donors (AD; blue filled bars) were assessed for percentages and counts (**A**; HD *n* = 20, AD *n* = 24), CD16 GMFI (**B**; HD *n* = 42, AD *n* = 24), CXC chemokine receptors CXCR1, CXCR2, and CXCR4 GMFI values (**C**; HD *n* = 50, AD *n* = 20), and MPO GMFI (**D**; HD *n* = 29, AD *n* = 16). In **A**, bar histogram represents neutrophil percentages (left Y axis) and symbols the absolute neutrophil counts (right Y axis) in HD (black bar and circle) and AD (blue filled bar and square). Data in **B** to **D** are presented as GMFI mean ± SD of different and unrelated donors. **E,** NET formation in HD (*n* = 10) or AD (*n* = 11) neutrophils after stimulation with 100 nM of PMA for 2 h. Neutrophils were stained with DAPI and analyzed by confocal microscopy (left panel), followed by manual counting of NET^+ neutrophils (middle panel) or automatic quantification of the DNA area (μm^2) using the DANA plug-in for ImageJ (right panel). Scale bar is 100 μm. Data are presented as mean ± SD of several donors. Significance of the differences between groups was calculated using Student's *t*-test; *ns*, Not significant.

Figure 7. Serum of allergic patients affects NET formation and migration of healthy human neutrophils. A, NET formation in HD neutrophils stimulated for 6 h with HD serum or AD serum, followed by stimulation with PMA (100 ng/mL) for 2 h and assessment. Neutrophils were stained and analyzed as described in **Fig. 6E**. Scale bar is 200 μm. Data are presented as mean ± SD of n = 10 unrelated donors. **B,** Chemotaxis of HD neutrophils toward a set concentration of CXCL8 (100 ng/mL) following stimulation for 2 h with HD serum (10% in RPMI 1640; grey bars) or AD serum (10% in RPMI 1640; red bars) together with an isotype-matched antibody (Ctrl; filled bars) or an anti-human CD124 antibody (5 μg/mL; Anti-CD124; striped bars). Shown are percentages of migrated neutrophils. Data are presented as mean ± SD of n = 10 unrelated donors. Significance of the difference between groups was calculated using Student's t-test (**A**) or one-way ANOVA (**B**); *ns*, Not significant.

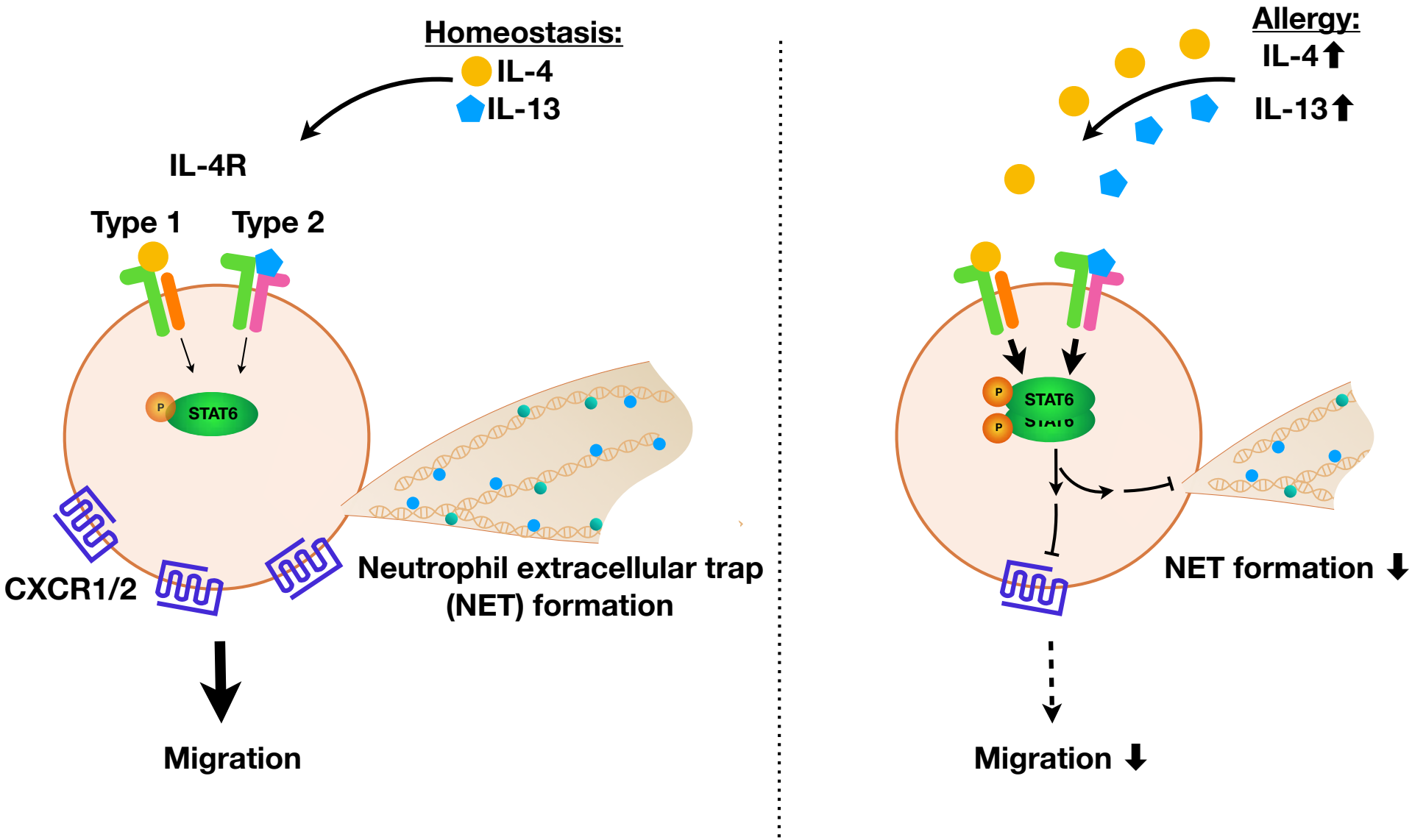

Homeostasis:
IL-4
IL-13
IL-4R
Type 1
Type 2
P
STAT6
CXCR1/2
Neutrophil extracellular trap
(NET) formation
Migration
Allergy:
IL-4⬆
IL-13⬆
P
STAT6
P
NET formation ⬇
Migration ⬇

Fig. 1

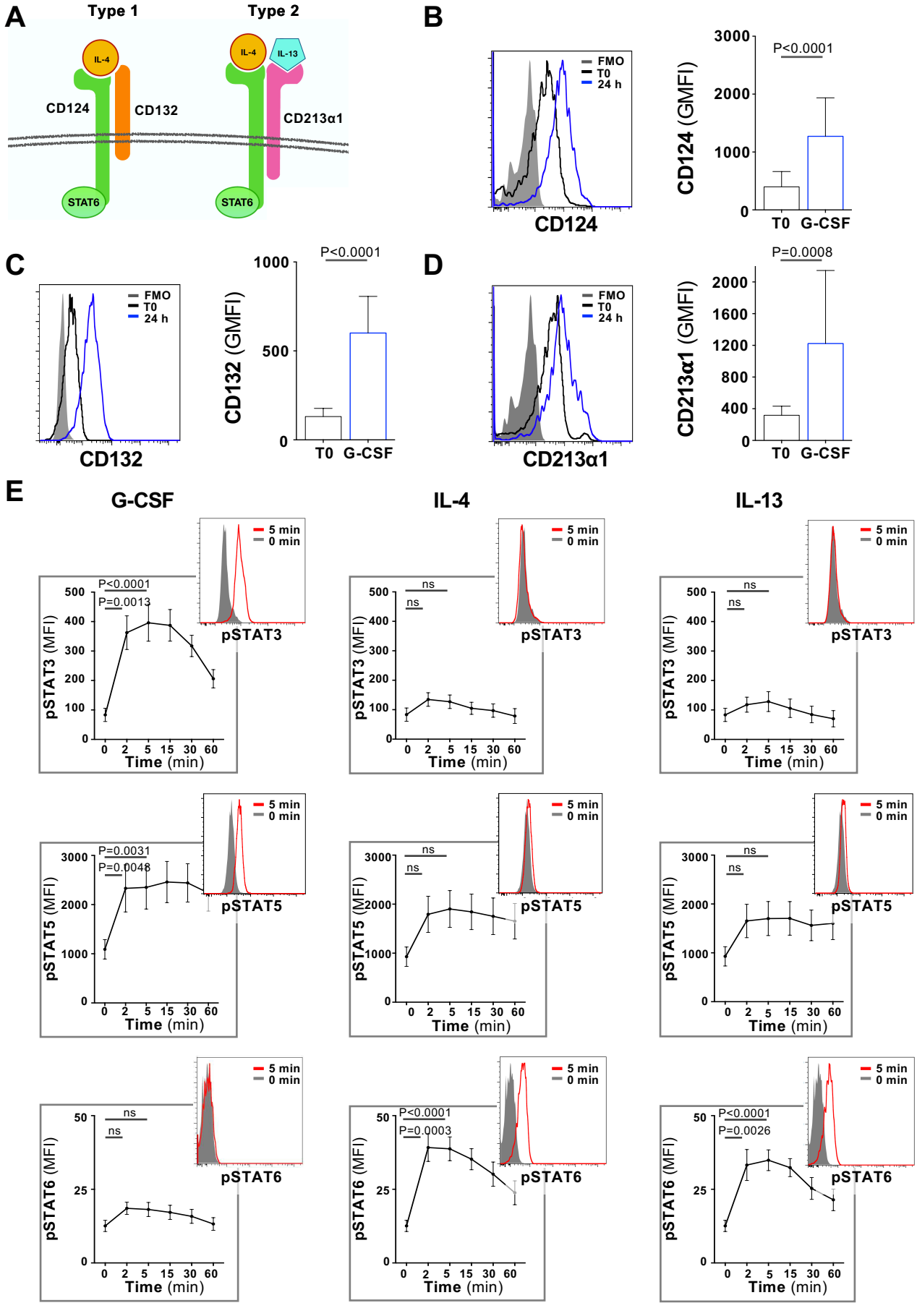
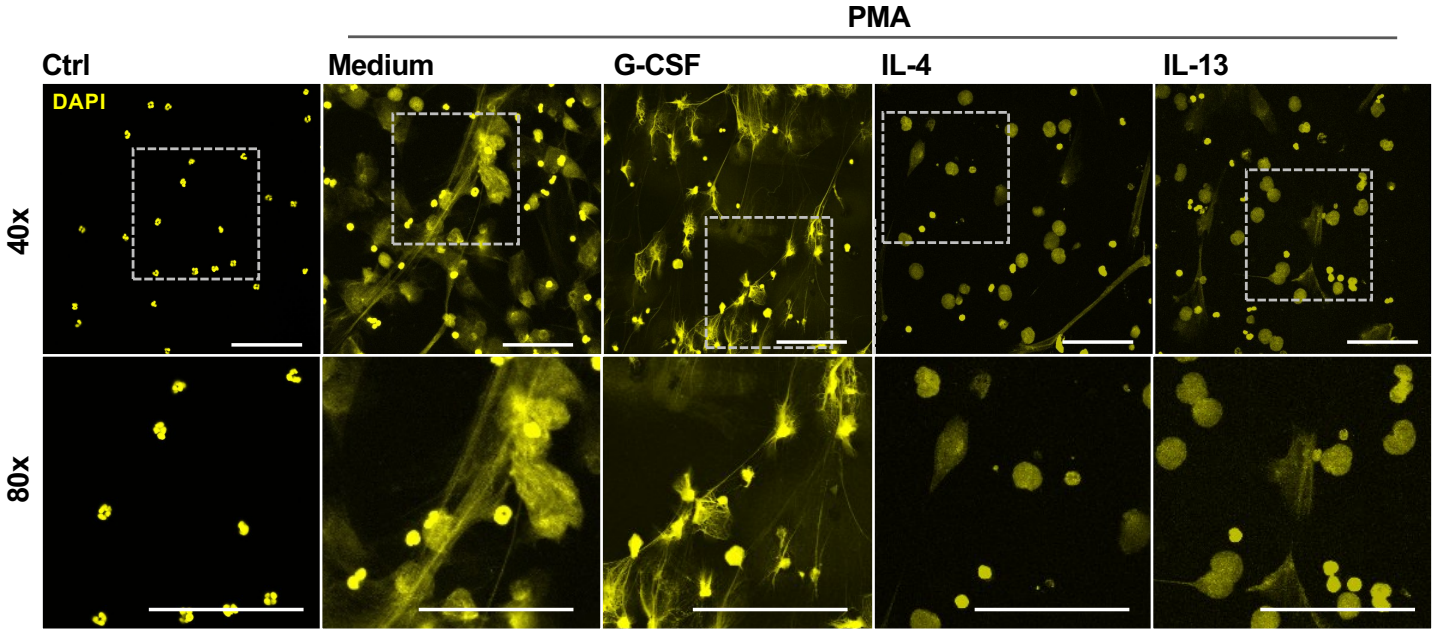
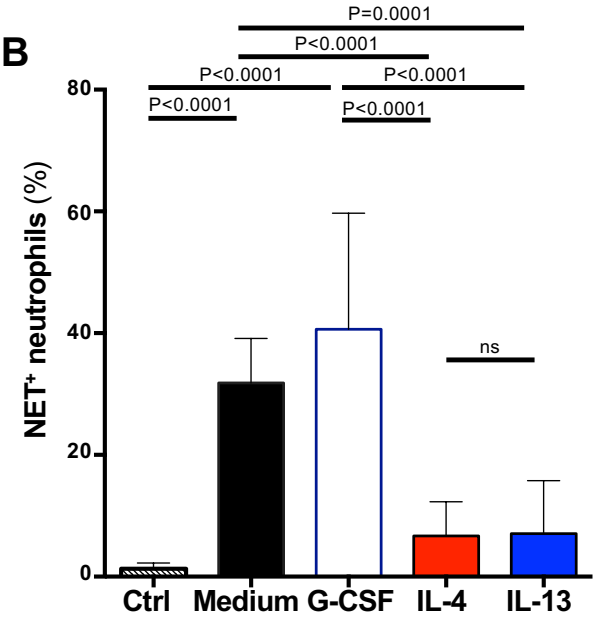

A
Type 1
Type 2
IL-4
IL-4
IL-13
CD124
CD132
CD213α1
STAT6
STAT6
B
FMO
T0
24 h
CD124
CD124 (GMFI)
P<0.0001
T0
G-CSF
C
FMO
T0
24 h
CD132
CD132 (GMFI)
P<0.0001
T0
G-CSF
D
FMO
T0
24 h
CD213α1
CD213α1 (GMFI)
P=0.0008
T0
G-CSF
E
G-CSF
IL-4
IL-13
5 min
0 min
pSTAT3
pSTAT3 (MFI)
P<0.0001
P=0.0013
ns
ns
pSTAT5
pSTAT5 (MFI)
P=0.0031
P=0.0048
ns
ns
pSTAT6
pSTAT6 (MFI)
ns
ns
P<0.0001
P=0.0003
P<0.0001
P=0.0026
Time (min)

Fig. 2

A


PMA
Ctrl
Medium
G-CSF
IL-4
IL-13
DAPI
40x
80x

B


P=0.0001
P<0.0001
P<0.0001
P<0.0001
P<0.0001
NET+ neutrophils (%)
80
60
40
20
0
ns
Ctrl
Medium
G-CSF
IL-4
IL-13

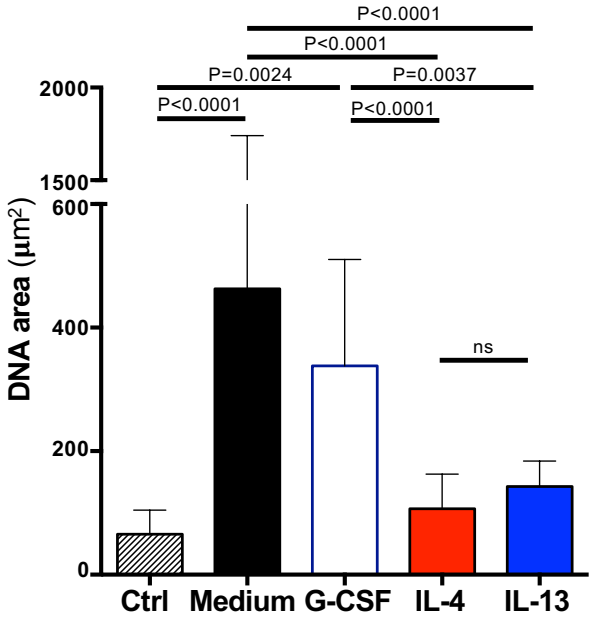

P<0.0001
P<0.0001
P=0.0024
P=0.0037
P<0.0001
P<0.0001
DNA area (µm2)
2000
1500
600
400
200
0
ns
Ctrl
Medium
G-CSF
IL-4
IL-13

Fig. 3

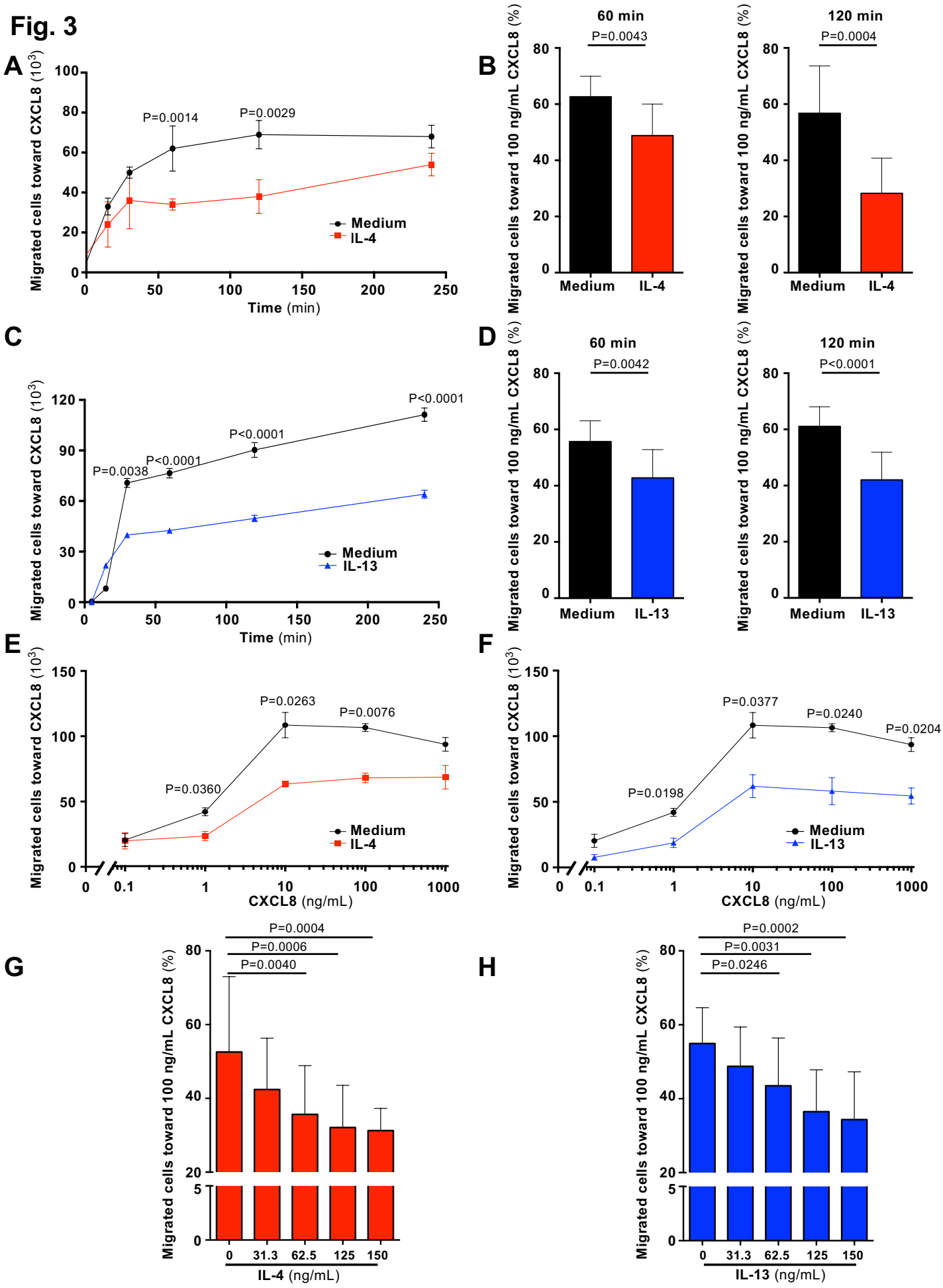

A
Migrated cells toward CXCL8 (10³)
P=0.0014
P=0.0029
Medium
IL-4
Time (min)
B
60 min
P=0.0043
120 min
P=0.0004
Migrated cells toward 100 ng/mL CXCL8 (%)
Medium
IL-4
C
P=0.0038
P<0.0001
P<0.0001
P<0.0001
Medium
IL-13
Time (min)
D
60 min
P=0.0042
120 min
P<0.0001
E
P=0.0360
P=0.0263
P=0.0076
Medium
IL-4
CXCL8 (ng/mL)
F
P=0.0198
P=0.0377
P=0.0240
P=0.0204
Medium
IL-13
CXCL8 (ng/mL)
G
P=0.0004
P=0.0006
P=0.0040
IL-4 (ng/mL)
H
P=0.0002
P=0.0031
P=0.0246
IL-13 (ng/mL)

Fig. 4

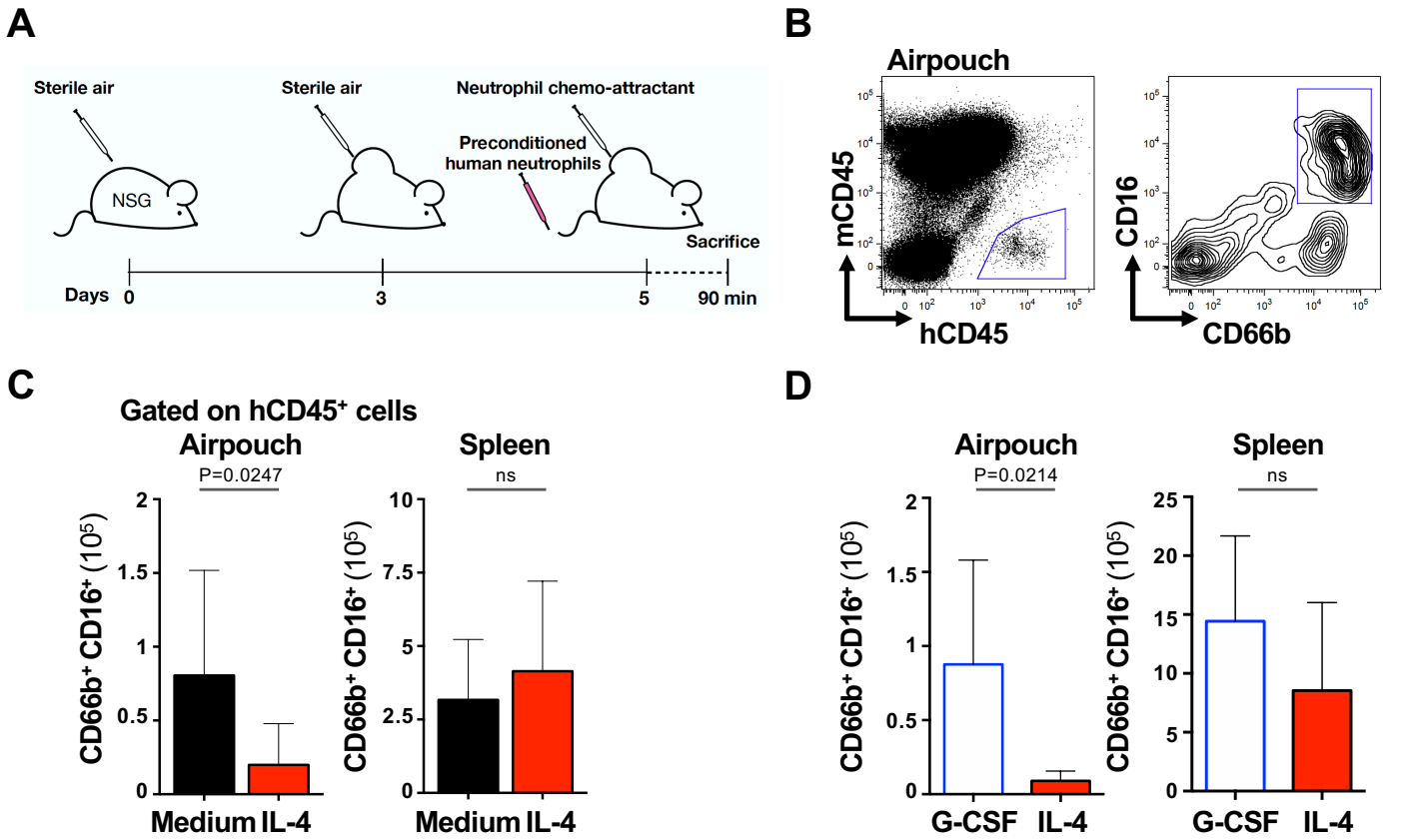

A
Sterile air
Sterile air
Neutrophil chemo-attractant
Preconditioned human neutrophils
NSG
Sacrifice
Days 0
3
5
90 min
B
Airpouch
mCD45
hCD45
CD16
CD66b
C
Gated on hCD45+ cells
Airpouch
P=0.0247
Spleen
ns
CD66b+ CD16+ (10^5)
Medium IL-4
D
Airpouch
P=0.0214
Spleen
ns
G-CSF IL-4

Fig. 5

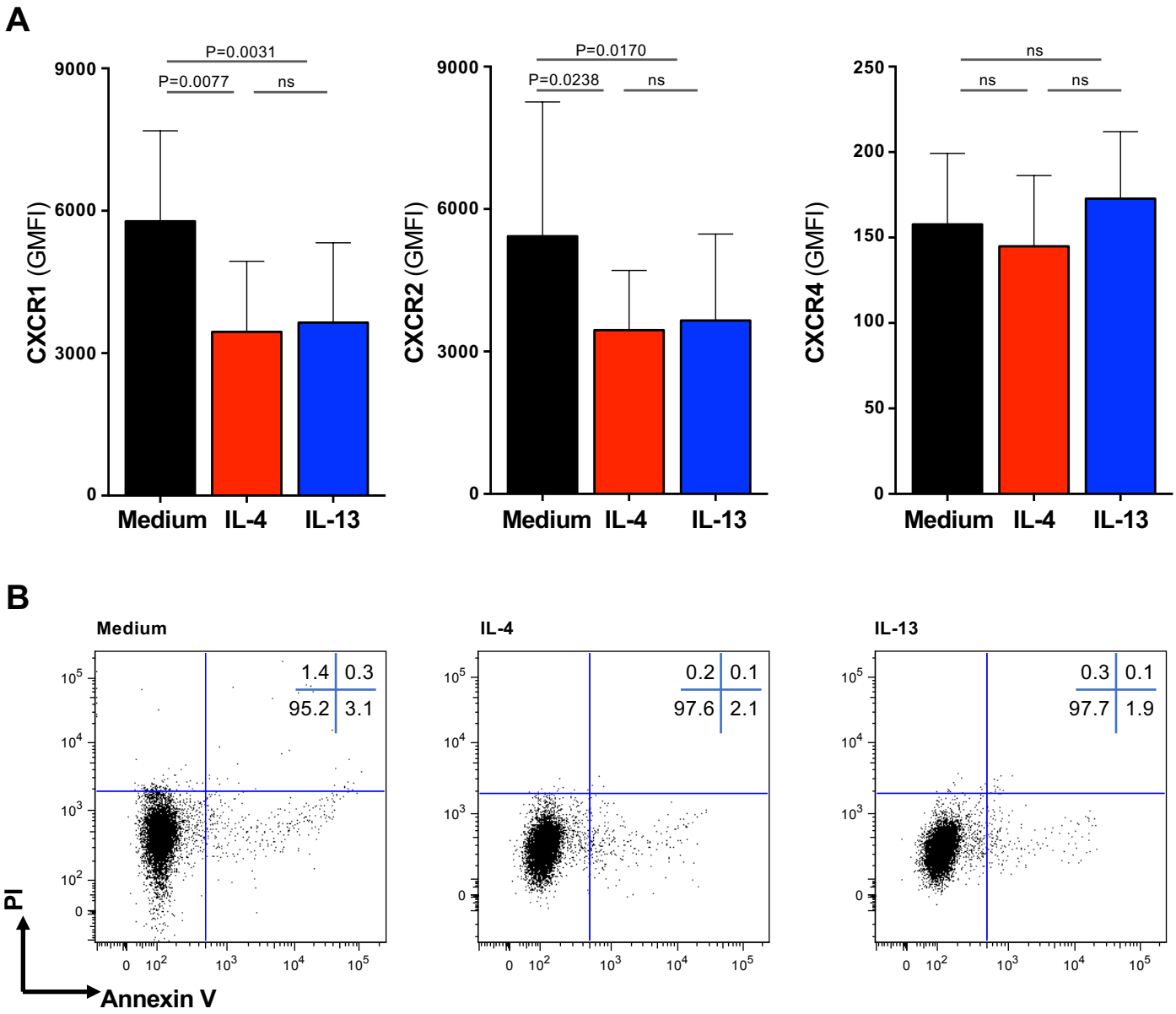

A
P=0.0031
P=0.0077
ns
9000
6000
3000
0
CXCR1 (GMFI)
Medium
IL-4
IL-13
P=0.0170
P=0.0238
ns
CXCR2 (GMFI)
ns
ns
ns
250
200
150
100
50
CXCR4 (GMFI)
B
Medium
IL-4
IL-13
1.4
0.3
95.2
3.1
0.2
0.1
97.6
2.1
0.3
0.1
97.7
1.9
PI
Annexin V

Fig. 6

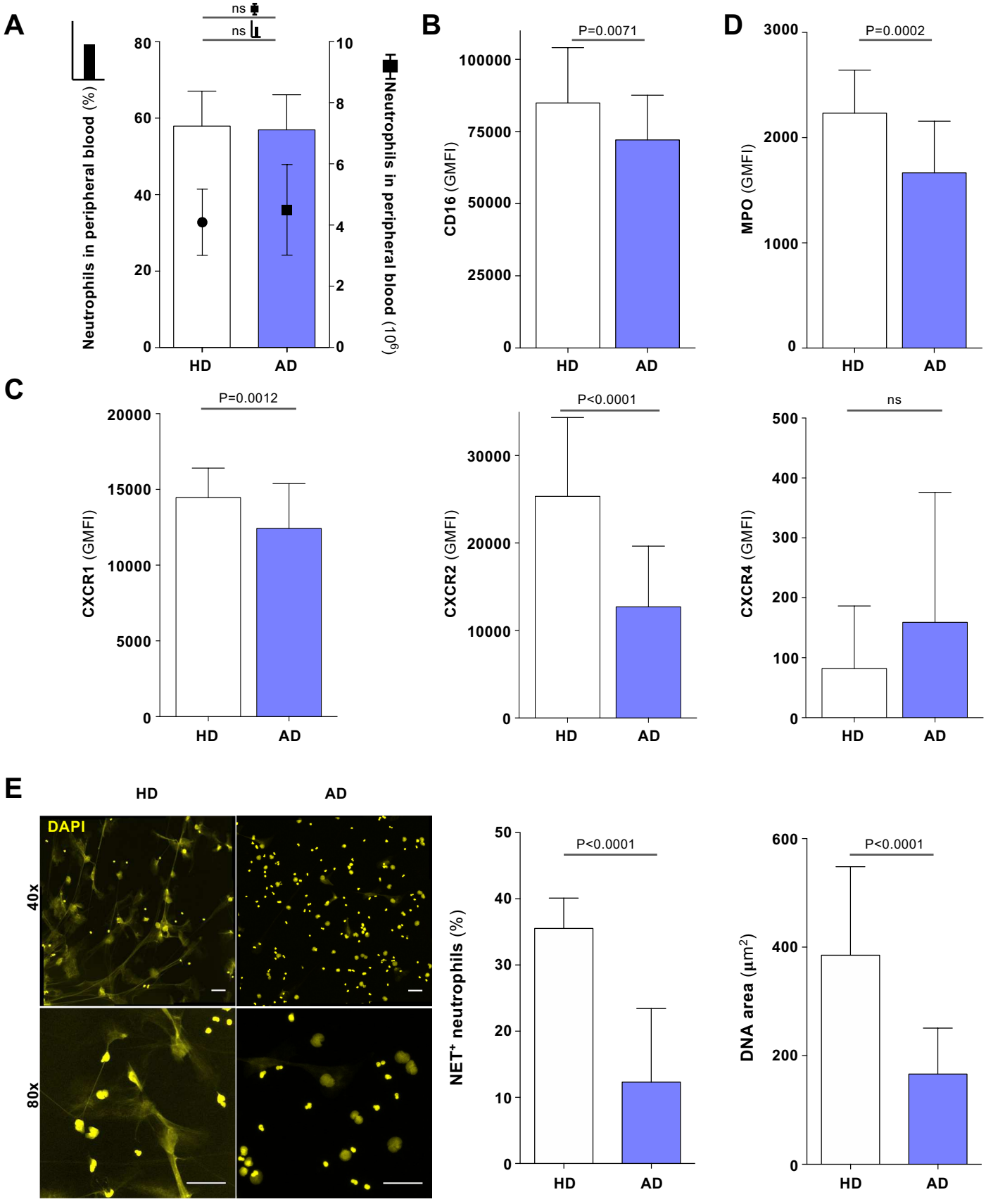

A
ns
ns
Neutrophils in peripheral blood (%)
H-Neutrophils in peripheral blood (10^9)
HD
AD
B
P=0.0071
CD16 (GMFI)
D
P=0.0002
MPO (GMFI)
C
P=0.0012
CXCR1 (GMFI)
P<0.0001
CXCR2 (GMFI)
ns
CXCR4 (GMFI)
E
HD
AD
DAPI
40x
80x
P<0.0001
NET+ neutrophils (%)
P<0.0001
DNA area (µm2)

Fig. 7

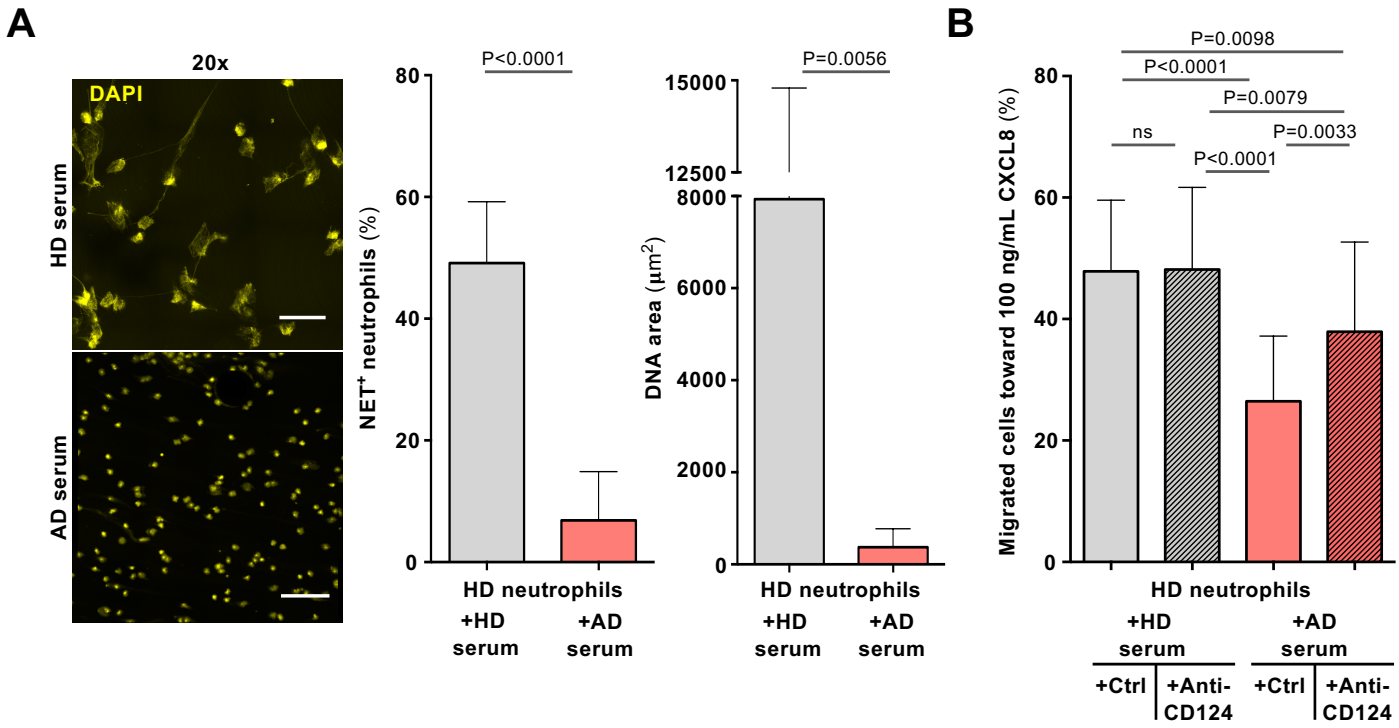

A
20x
DAPI
HD serum
AD serum
P<0.0001
NET+ neutrophils (%)
80
60
40
20
0
HD neutrophils
+HD serum
+AD serum
P=0.0056
DNA area (µm2)
15000
12500
8000
6000
4000
2000
0
HD neutrophils
+HD serum
+AD serum
B
P=0.0098
P<0.0001
P=0.0079
ns
P=0.0033
P<0.0001
Migrated cells toward 100 ng/mL CXCL8 (%)
80
60
40
20
0
HD neutrophils
+HD serum
+AD serum
+Ctrl
+Anti-CD124
+Ctrl
+Anti-CD124

Fig. E1

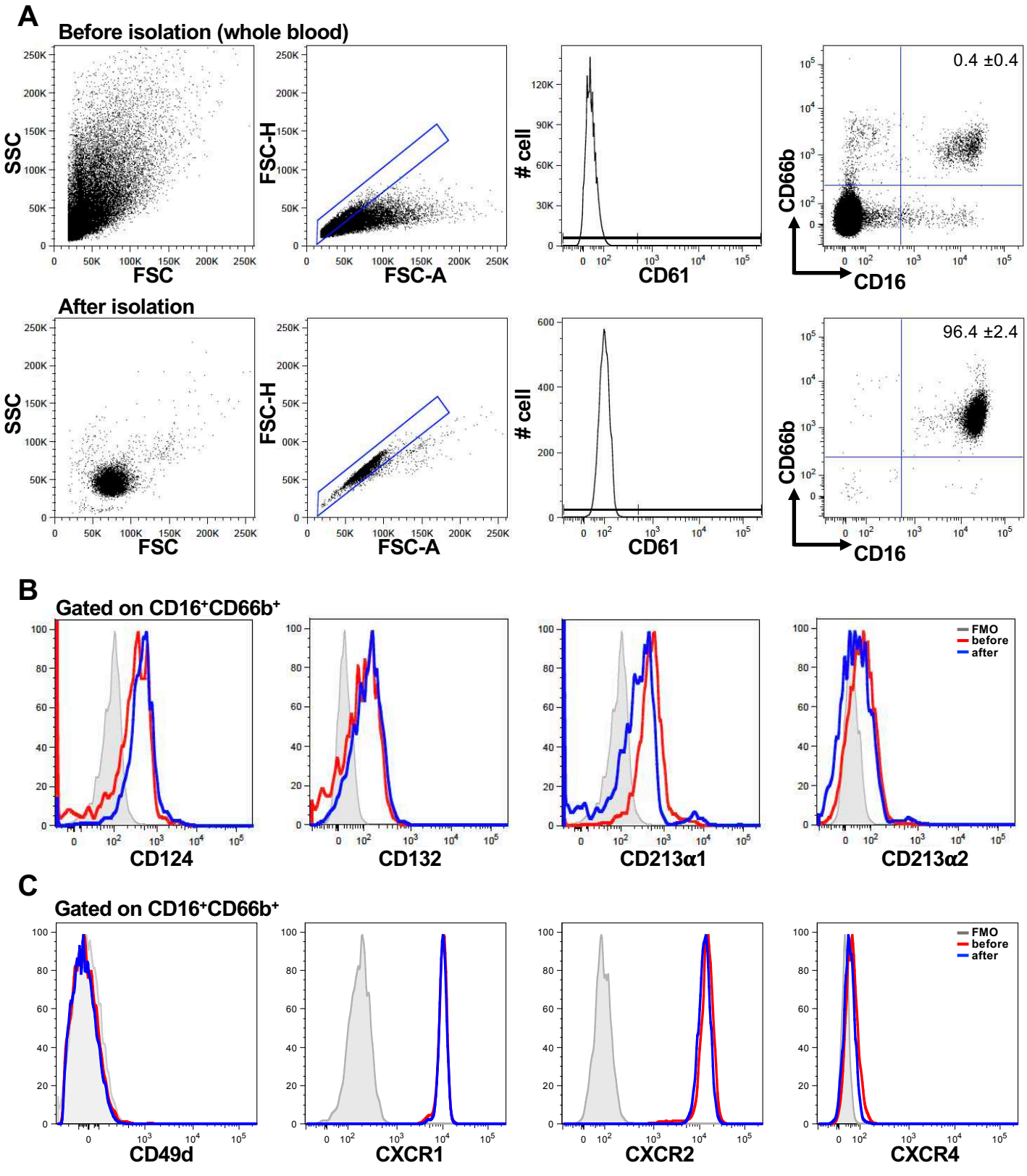

A
Before isolation (whole blood)
SSC
FSC
FSC-H
FSC-A
cell
CD61
CD66b
CD16
0.4 ±0.4
After isolation
96.4 ±2.4
B
Gated on CD16⁺CD66b⁺
CD124
CD132
CD213α1
CD213α2
FMO
before
after
C
Gated on CD16⁺CD66b⁺
CD49d
CXCR1
CXCR2
CXCR4

Fig. E2

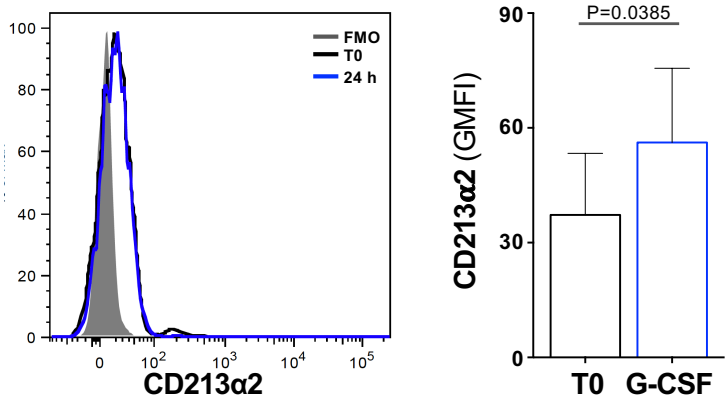

FMO
T0
24 h
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
CD213α2
P=0.0385
90
60
30
0
CD213α2 (GMFI)
T0
G-CSF

Fig. E3

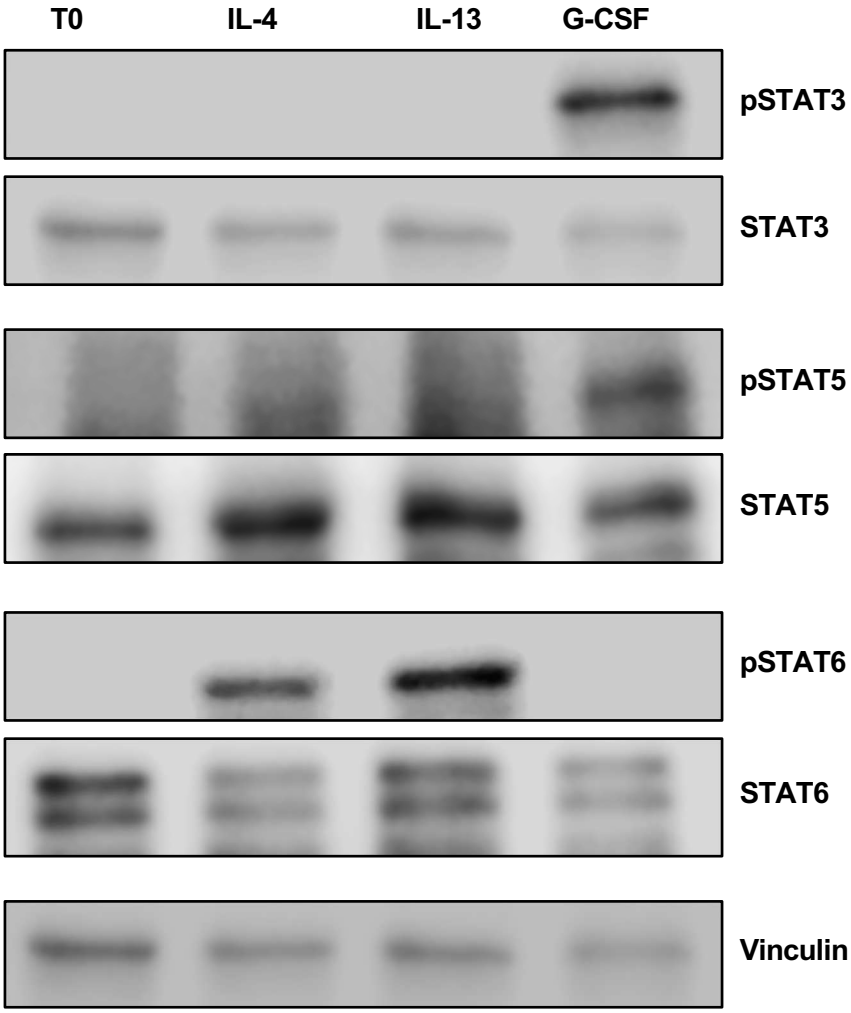
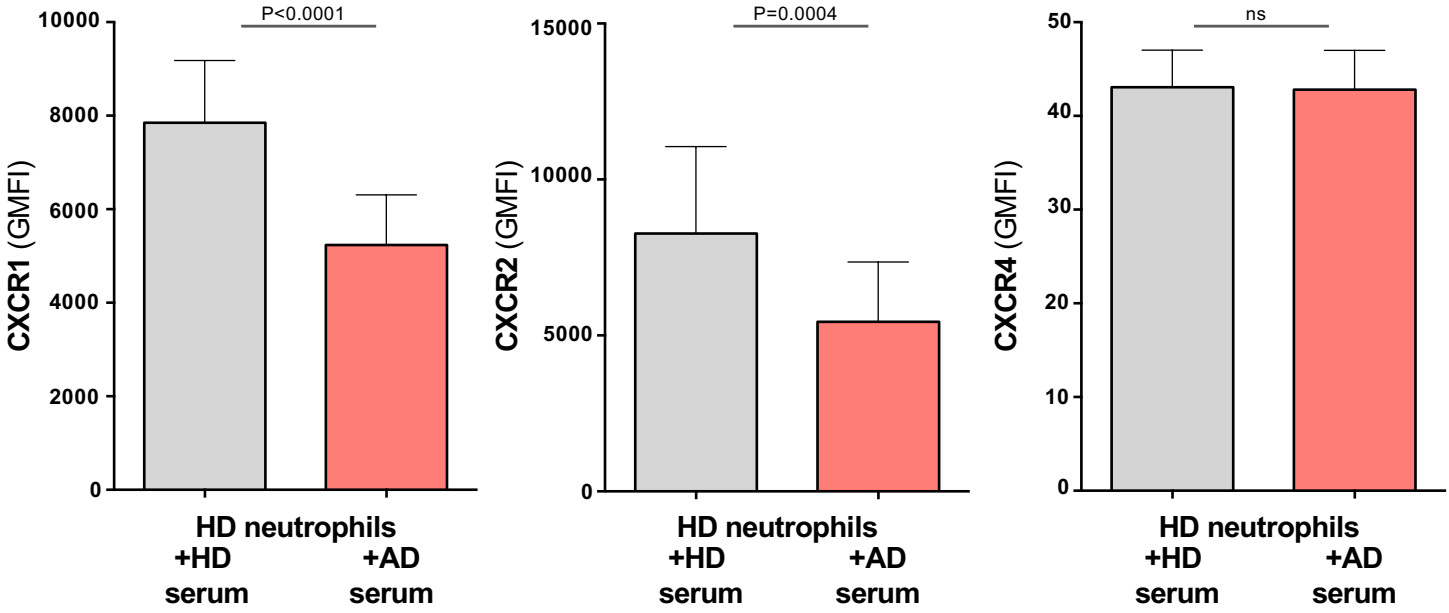

T0
IL-4
IL-13
G-CSF
pSTAT3
STAT3
pSTAT5
STAT5
pSTAT6
STAT6
Vinculin

Fig. E4


P<0.0001
10000
8000
6000
4000
2000
0
CXCR1 (GMFI)
HD neutrophils
+HD serum
+AD serum
P=0.0004
15000
10000
5000
0
CXCR2 (GMFI)
HD neutrophils
+HD serum
+AD serum
ns
50
40
30
20
10
0
CXCR4 (GMFI)
HD neutrophils
+HD serum
+AD serum